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The *Paxillus involutus* (Boletales, Paxillaceae) complex in Europe: Genetic diversity and morphological description of the new species *Paxillus cuprinus*, typification of *P. involutus* s.s., and synthesis of species boundaries

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Highlights

- *Paxillus involutus* is a model species for research on ectomycorrhizal fungi.
 - Species boundaries in *P. involutus* complex were explored.
 - A phylospecies is taxonomically described and named *Paxillus cuprinus* sp. nov.
 - Level of genetic diversity correlates with morphological and ecological traits.
 - Genetic divergence between species is below the 3% cut-off used in barcoding.
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Abstract

Paxillus involutus is a model species for ecological or physiological studies of ectomycorrhizal agaricomycetes. Three to six groups or species linked to it have been ecologically and morphologically distinguished. Phylogenetic studies have revealed the existence of four species in Europe: *Paxillus ammoniavirescens*, *Paxillus obscurisporus*, *P. involutus*, and a fourth as yet not described species. We studied 47 collections from 24 French and Italian locations, supplemented with GenBank data, in order to genetically and taxonomically delineate these species. Phylogenetic analyses of three nuclear DNA regions (rDNA internal transcribed spacer (ITS), *tef1-α*, and *gpd*)

confirmed the four European species. Morphology, culture, and ecology features allowed us to delineate species boundaries and to describe the fourth species we named *Paxillus cuprinus* since it turns coppery with age. As there is no existing original herbarium specimen for *P. involutus*, one of our collections was chosen as the epitype. The low genetic diversity found in *P. cuprinus* correlates with stable morphological traits (basidiome colour, ovoid–amygdaliform spores with an apical constriction) and with ecological preferences (association with *Betulaceae* in open and temperate areas). In contrast, *P. ammoniavirescens* is characterized by a high genetic diversity and a high variation of its morphological and ecological features.

Keywords

- Barcoding;
 - Ecological traits;
 - Morphometry;
 - Ectomycorrhizal fungi;
 - Taxonomy
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Introduction

Members of the genus *Paxillus* (Agaricomycota, Boletales, Paxillaceae) are ectomycorrhizal fungi associated with various woody host plant species including hardwood and coniferous trees. They are commonly encountered in a wide range of ecosystems and habitats through the northern hemisphere.

Paxillus is a taxon of importance for academic research, as shows a bibliographic search done in early 2013 through the international database ISI Web of Science (<http://www.isiknowledge.com/>) among the scientific literature dealing with fungi. The item *Paxillus* was recorded in the title of 152 scientific publications and in the topic of at least 690 different published works originating from about 60 different countries. The species *Paxillus involutus* accounted for most of these records. It is in the top four of ectomycorrhizal species used as models for scientific studies that have significantly contributed to advances in the knowledge of ectomycorrhizal symbiosis functioning. The genomes of *P. involutus* and *Paxillus rubicundulus* strains have been entirely sequenced ([Martin et al., 2011](#) and [Martin and Bonito, 2012](#)) underlining scientists' keen interest in the genus. Regarding human health, fatal outcomes due to immune haemolysis ([Winkelmann et al., 1986](#) and [Anthrowiak et al., 2003](#)) have been reported after ingestion of *P. involutus*. However, assessments whether *P. involutus* and other closely related species are harmful or not vary depending on different observers' experiences in different countries. Differences in 'toxicity' assessments can depend on the speciation pattern. After all, there is no type reference sequence for *P. involutus* and the concept of this species is based on [Batsch's icon \(1786\)](#).

In Europe, the genus *Paxillus* is divided into two groups: the *P. involutus* complex and *P. rubicundulus*. The latter is mainly encountered in wetland habitats and exclusively associated with alder trees (*Alnus* spp., *Betulaceae*) ([Orton 1969](#)) whereas species of the *P. involutus* complex are found in more diverse habitats and associated with several tree species. Thus, host specificity is a useful trait to distinguish *P. rubicundulus* but species recognition within the *P. involutus* complex remains very difficult. Mycologists have long suspected that the name *P. involutus* encompasses different species ([Fries, 1985](#), [Hahn and Agerer, 1999](#), [Jarosch and Bresinsky, 1999](#) and [Bresinsky, 2006](#)). Thus, mating tests performed with Swedish isolates revealed three intersterility groups ([Fries](#)

[1985](#)). Although these groups were not correlated with clear-cut morphological characters, ecological traits distinguished them: group 1 was found in forest habitats whereas groups 2 and 3 were found in city parks and gardens ([Fries 1985](#)). On the basis of a morphological study, [Hahn & Agerer \(1999\)](#) recognized four European species in the *P. involutus* complex: *P. involutus*, *Paxillus validus*, *Paxillus albidulus*, and *Paxillus obscurisporus*. Finally, a phylogenetic study based on five genes unambiguously revealed four genetic lineages within the *P. involutus* complex ([Hedh et al. 2008](#)). More recently, [Vellinga et al. \(2012\)](#) revisited the *P. involutus* complex with a larger number of samples originating from a broader geographic range, including Europe and North America, and also found the same four genetic lineages. Two of these lineages corresponded to the described morphological species *P. obscurisporus* and *Paxillus ammoniavirescens* (syn. *P. validus*), a third one was assigned to *P. involutus* s.s. ([Hedh et al. 2008](#)), and the fourth lineage, referred to as PS IV and as *P. involutus* II by [Hedh et al. \(2008\)](#) and [Vellinga et al. \(2012\)](#) respectively, remains a phylopecies without an established correspondence with any described morphospecies. All these species can be encountered in highly diverse environments and locations and may thus be included in molecular environmental studies. Therefore it appears useful to remove any ambiguity regarding them.

Exploration of France led us to observe a large and unusual variation among *Paxillus* specimens and to suspect the presence of the not yet described phylopecies reported by [Hedh et al. \(2008\)](#) and [Vellinga et al. \(2012\)](#). This paper aims to describe the species corresponding to PS IV/*P. involutus* II and to study various aspects (genetic, morphological, and ecological) of the diversity of the *P. involutus* complex as exhaustively as possible, based on our collections and on data from the literature, in order to establish a framework to delineate these *Paxillus* species. The objectives were (1) to assign specimens to lineages and to study genetic variation within and among the lineages in order to determine a phylogenetic cut-off for species delimitation (2) to identify key differentiating morphological features between species (3) to reveal the ecological preferences of each species.

Materials and methods

Collections and ecological features

From 2008 to 2012, we obtained 47 collections of *Paxillus* basidiomes that matched with the macroscopic characteristics of the *Paxillus involutus* complex, from 23 locations in France and one in Italy ([Table 1](#)). After morphological investigations, the collected specimens were dried, except for a small amount of fresh tissues that was frozen at -20°C . During the collecting step, an environment type was assigned to each collection. Environment was defined as ‘closed’ (forest) or ‘open’, in natural conditions (forest edge, roadside in forest, river bank) and in urban areas (public lawn, city park including garden and car park) ([Table 1](#)). Available environmental data from the literature ([Table 2](#)) were also taken into account and considered as ‘closed’ environments (forest) or ‘open’ environments (park, lawn, pasture). As much as possible, potential ectomycorrhizal host plants present in the surroundings of the collecting places were recorded ([Table 1](#)).

Table 1.

Collections and sequenced isolates included in the study. In bold: type collection and specimens used for spore size statistical analysis.

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	P	S ^a	Herbarium number	Accession number		
									number	ITS	<i>tef</i>	<i>gpd</i>
ALB08	2008	Amélie-les-Bains, Pyrénées-Orientales, France	River bank	<i>Populus, Alnus</i>	230 m	ALB08.1	P	A			KF26 1492	KF26 1423
Alz12	2012	Alzen, Ariège, France	Forest	<i>Betula, Fagus</i>	720 m	Alz12.1	PI	B121123			KF26 1494	KF26 1427
Arf08	2008	Arfons, Tarn, France	nd	nd	754 m	Arf08.1	PI	MN08.1			KF26 1355	KF26 1495
												KF26 1428
Arf09	2009	Arfons, Tarn, France	nd	nd	754 m	Arf09.1	P	C	MN09.1			KF26 1357
												KF26 1497
												KF26 1430
Arf12	2012	Arfons, Tarn, France	nd	nd	754 m	Arf09.2	PI	MN09.2			KF26 1358	KF26 1498
												KF26 1431
Auc12	2012	Auch, Gers, France	River bank	<i>Alnus</i>	133 m	Auc12.1	P	R	B121014			KF26 1361
												KF26 1499
												KF26 1434
Auz08	2008	Auzeville-Tolosane, Haute-Garonne, France	City park	<i>Betula, Pinus</i>	153 m	Auz08.15	P	A			KF26 1362	KF26 1500
												KF26 1435

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	Herbarium		Accession number		
							P ^a	number ^b	ITS	tef	gpd
						Auz08.33	P A				KF26 1436
Bel09	2009	Belcaire, Aude, France	Forest	<i>Picea</i>	1050 m	Bel09.1	PI		KF26 1363	KF26 1501	KF26 1437
	2010	Belcaire, Aude, France	Forest	<i>Abies</i>	1050 m	Bel10.2	PI		KF26 1364	KF26 1502	
Bel10						Bel10.3	PI		KF26 1365	KF26 1503	KF26 1438
						Bel10.4	PI		KF26 1366	KF26 1504	
Bel12	2012	Belcaire, Aude, France	Public lawn	<i>Betula</i>	1050 m	Bel12.1	P O	B12102 9	KF26 1367	KF26 1505	KF26 1439
Bou12	2012	Bouconne, Haute-Garonne, France	Forest edge	<i>Quercus</i>	240 m	Bou12.1	P A	B12103 2		KF26 1506	KF26 1440
Can12.F	2012	Cantaous, Hautes-Pyrénées, France	Forest	<i>Picea</i>	590 m	Can12.1	PI	B12114 0.1	KF26 1368	KF26 1507	KF26 1441
						Can12.2	PI	B12114 0.2	KF26 1369	KF26 1508	KF26 1442
Can12.R	2012	Cantaous, Hautes-Pyrénées, France	Roadside in forest	<i>Corylus</i>	590 m	Can12.3	P C	B12100 7	KF26 1370	KF26 1509	KF26 1443
Car10	2010	Cardeilhac, Haute-Garonne, France	nd	nd	491 m	Car10.1	P C		KF26 1371	KF26 1510	KF26 1444
						Car10.2	PI		KF26 1372	KF26 1511	

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	P	S ^a	Herbarium number ^b	Accession number		
										ITS	<i>tef</i>	<i>gpd</i>
						Car10.3	PI			KF26 1373	KF26 1512	KF26 1445
						Car10.4	PI			KF26 1374	KF26 1513	
						Car10.5	PI			KF26 1375	KF26 1514	KF26 1446
Car12	2012	Cardeilhac, Haute-Garonne, France	Roadside in forest	<i>Quercus</i>	491 m	Car12.1	P	B12110A 1		KF26 1376		KF26 1447
Cas09	2009	Castanet-Tolosan, Haute-Garonne, France	City park	<i>Betula</i>	156 m	Cas09.1	P	C		KF26 1377	KF26 1515	KF26 1448
						Cas09.3	P	C		KF26 1378	KF26 1516	KF26 1449
Cas10	2010	Castanet-Tolosan, Haute-Garonne, France	City park	<i>Betula</i>	156 m	Cas10.3	P	C	TL127-0002	KF26 1379	KF26 1517	KF26 1450
Cas12.A	2012	Castanet-Tolosan, Haute-Garonne	City park	<i>Betula</i>	156 m	Cas12.1	P	B12100A 6		KF26 1380	KF26 1518	KF26 1451
Cas12.B	2012	Castanet-Tolosan, Haute-Garonne, France	City park	<i>Betula</i>	156 m	Cas12.4	P	C		KF26 1381	KF26 1519	KF26 1452
						Cas12.5	P	C 5	B12100	KF26 1382		KF26 1453
Cer09	2009	Céret, Pyrénées	River bank	<i>Populus, Alnus</i>	113 m	Cer09.15	P	A		KF26 1383	KF26 1520	KF26 1454

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	P	Herbarium number	Accession number		
									ITS	tef	gpd
Eau11	2011	s-Orientales, France									
		Eauzes, Gers, France	River bank	<i>Alnus</i> , <i>Salix</i>	145 m	Eau11.1	P C 1	B11110	KF26 1384	KF26 1521	KF26 1455
						Eau11.2	P A 2	B11110	KF26 1385	KF26 1522	KF26 1456
Hec12	2012	Hèches, Hautes-Pyrénées, France	Forest edge	<i>Corylus</i>	630 m	Hec12.1	P O 2	B12101	KF26 1386	KF26 1523	KF26 1457
Lab12	2012	Labège, Haute-Garonne, France	City park	<i>Populus</i>	145 m	Lab12.1	P A			KF26 1524	KF26 1458
Lil12	2012	Lille, Nord, France	City park	<i>Chamaecyparis</i> , <i>Betula</i>	40 m	Lil12.1	P C		KF26 1387	KF26 1525	KF26 1459
Lou12	2011	Loubersan, Gers, France	Roadside in forest	<i>Quercus</i>	260 m	Lou11.1	P A 8	B11111	KF26 1388	KF26 1526	KF26 1460
PM10.A	2010	Prats-de-Mollo, Pyrénées-s-Orientales, France	Roadside in forest	<i>Betula</i>	925 m	PM10.A1	P C 1	CA10.A	KF26 1389	KF26 1527	KF26 1461
						PM10.A2	P C 2	CA10.A	KF26 1390	KF26 1528	KF26 1462
						PM10.A4	P C 4	CA10.A	KF26 1391	KF26 1529	KF26 1463
PM10.C	2010	Prats-de-Mollo, Pyrénées-s-Orientales	Forest edge	<i>Pinus</i> , <i>Betula</i>	992 m	PM10.C1	P C 1	CA10.B	KF26 1392	KF26 1530	KF26 1464

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	P	Herbarium number	Accession number		
									ITS	tef	gpd
PM10.B	2010	es, France Prats-de-Mollo, Pyrénées-Orientales, France	Forest	<i>Pinus, Betula</i>	998 m	PM10.B2	PI	CA10.B2	KF26 1393	KF26 1531	
								CA10.B3	KF26 1394	KF26 1532	
Pou09	2009	Pouvourville, Haute-Garonne, France	City park	<i>Cedrus</i>	235 m	Pou09.1	P A		KF26 1395	KF26 1533	KF26 1465
						Pou09.2	P A				KF26 1466
						Pou09.3	P A				KF26 1467
Pun11	2011	Puntous, Hautes-Pyrénées, France	Roadside in forest	<i>Quercus</i>	310 m	Pun11.1	P A	B110905	KF26 1396	KF26 1534	KF26 1468
Ram10	2010	Ramonville-Saint-Agne, Haute-Garonne, France	City park	<i>Cedrus</i>	160 m	Ram10.1	P A		KF26 1397	KF26 1535	KF26 1469
						Ram10.2	P A		KF26 1398	KF26 1536	
						Ram10.3	P A		KF26 1399	KF26 1537	
Ram11	2011	Ramonville-Saint-Agne, Haute-	City park	<i>Betula</i>	160 m	Ram11.2	P A	PTC11.2	KF26 1400	KF26 1538	KF26 1470

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	P S ^a	Herbarium	Accession number		
								number _b	ITS	tef	gpd
Sau09	2009	Garonne, France Saurat, Ariège, France	Forest	<i>Abies</i> , <i>Picea</i>	1180 m	Sau09.1	PI	CP09.1	KF26 1401		KF26 1471
Sau10.F	2010	Saurat, Ariège, France	Forest	<i>Abies</i>	1180 m	Sau10.1	PI	CP10.1	KF26 1402	KF26 1539	KF26 1472
						Sau10.2	PI	CP10.2	KF26 1403	KF26 1540	
Sau10.R	2010	Saurat, Ariège, France	Roadside in forest	<i>Betula</i> , <i>Alnus</i>	1150 m	Sau10.3	P C	CP10.3	KF26 1404	KF26 1541	
Sau11.F	2011	Saurat, Ariège, France	Forest	<i>Abies</i> , <i>Picea</i>	1180 m	Sau11.1	PI	CP11.1	KF26 1405	KF26 1542	KF26 1473
						Sau11.3	PI	CP11.3	KF26 1406	KF26 1543	KF26 1474
						Sau11.6	PI	CP11.6	KF26 1407		
Sau12.F	2012	Saurat, Ariège, France	Forest	<i>Abies</i>	1180 m	Sau12.7	PI	CP12.7	KF26 1408		KF26 1475
Sau12.R	2012	Saurat, Ariège, France	Roadside in forest	<i>Betula</i>	1150 m	Sau12.4	P A	CP12.4	KF26 1409		KF26 1476
SCV09	2009	Sainte-Croix-Volvestre, Ariège, France	Forest	<i>Abies</i>	380 m	SCV09.1	PI		KF26 1410		KF26 1477
						SCV09.5	PI				KF26 1478
SCV11	2011	Sainte-Croix-Volvestre, Ariège, France	Forest	<i>Abies</i>	380 m	SCV11.1	PI	B111013	KF26 1411	KF26 1544	KF26 1479
SVB08	2008	Saint-Vincent-en-	Public lawn	<i>Betula</i>	210 m	SVB08.1	P C			KF26 1545	KF26 1480

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	P S ^a	Herbarium number ^b	Accession number		
									ITS	<i>tef</i>	<i>gpd</i>
		Bresse, Saône-et-Loire, France				SVB0 8.2	P C		KF26 1412	KF26 1546	KF26 1481
						SVB0 8.3	P C			KF26 1547	KF26 1482
						SVB0 8.4	P C			KF26 1548	KF26 1483
Tou02	2002	Toulouse, Haute-Garonne, France	City park	<i>Corylus</i>	150 m	Tou02 .1	P C		KF26 1413		
Tou08	2008	Toulouse, Haute-Garonne, France	City park	<i>Pinus</i>	150 m	Tou08 .5	P A	UPS08. 5	KF26 1414	KF26 1549	KF26 1485
						Tou08 .23	P A	UPS08. 23			KF26 1484
Tou08 .C	2008	Toulouse, Haute-Garonne, France	City park	<i>Cedrus</i>	150 m	Tou08 .C	P A	UPS08. C	KF26 1415	KF26 1550	KF26 1486
Tou11 .A	2011	Toulouse, Haute-Garonne, France	City park	<i>Populus</i>	150 m	Tou11 .1	P A	UPS11. 1	KF26 1416		
Tou11 .B				<i>Cedrus</i>		Tou11 .7	P A	UPS11. 7	KF26 1417		KF26 1487
						Tou11 .8	P A	B11111 6	KF26 1418		KF26 1488
Tou11 .C	2011	Toulouse, Haute-	City park	<i>Cedrus, Betula</i>	150 m	Tou11 .9	P C	B11111 4	KF26 1419	KF26 1551	KF26 1489

Collection	Year of sampling	Location	Environment type	Recorded trees	Altitude	Isolates	PS ^a	Herbarium number ^b	Accession number		
									ITS	<i>tef</i>	<i>gpd</i>
Tou12	2012	Garonne, France Toulouse, Haute-Garonne, France Le Vernet,	City park	<i>Populus</i>	150 m	Tou12.1	P A 1	UPS12.	KF26 1420	KF26 1552	KF26 1490
Ve08	2008	Haute-Garonne, France Vezzano, Reggio nell'Emilia, Italy	River bank	<i>Alnus</i>	167 m	Ve08.2h10	P R		KF26 1421	KF26 1553	KF26 1491
Vez12	2012	Reggio nell'Emilia, Italy	Roadside in forest	<i>Ostrya</i> , <i>Quercus</i> , <i>Castanea</i>	500 m	Vez12.1	P C	TO_AV P164, GS10076	KF26 1422		

nd: not determined.

a

PS = phylogenetic species; PA = *Paxillus ammoniavirescens*; PC = *Paxillus cuprinus*; PI = *Paxillus involutus*; PO = *Paxillus obscurisporus*; PR = *Paxillus rubicundulus* (outgroup).

b

When different of the isolate name.

Table 2.

Sequences from GenBank included in this study and information about the specimens sequenced. Type specimens are in bold.

Accession number	Locus	PS ^a	Country	Recorded trees	Environment type	Herbarium number	Reference
AF104991	ITS	PC	Germany	<i>Alnus</i>	–	LHPfil1	Pritsch <i>et al.</i> 2000
AF167690	ITS	PO	Germany	<i>Tilia</i>	Park	Pi1	Jarosch & Bresinsky 1999

Accession number	Locus	PS ^a	Country	Recorded trees	Environment type	Herbarium number	Reference
AF167691	ITS	PO	Germany	<i>Corylus</i>	Park	Pi12	Jarosch & Bresinsky 1999
AF167692	ITS	PO	Germany	<i>Quercus</i>	Park	PiM1	Jarosch & Bresinsky 1999
AF167693	ITS	PO	Germany	<i>Betula</i>	Park	PiM4	Jarosch & Bresinsky 1999
AF167694	ITS	PO	Germany	<i>Populus</i>	Park	PiM2	Jarosch & Bresinsky 1999
AY585910	ITS	PO	Sweden	<i>Populus, Fagus</i>	Public lawn	SE03071001	Hedh et al. 2008
AY585911	ITS	PO	Sweden	<i>Populus, Cornus, Tilia, Fagus</i>	Public lawn	SE03-07-1622	Hedh et al. 2008
AY585913	ITS	PI	Scotland	<i>Betula</i>	–	ATCC 200175	Hedh et al. 2008
AY585915	ITS	PA	France	<i>Populus</i>	–	Nau	Hedh et al. 2008
AY585917	ITS	PA	France	<i>Quercus</i>	–	Maj	Hedh et al. 2008
AY585921	ITS	PC	Sweden	<i>Fagus, Betula</i>	Pasture	SE03100501	Hedh et al. 2008
AY585922	ITS	PC	Sweden	<i>Picea, Betula</i>	Unmanaged lawn	HW03092501	Hedh et al. 2008
DQ457629	<i>tef</i>	PO	China	<i>Pinus, Betula, Juglans</i>	Mountain	AFTOL-ID 715	Binder & Hibbett 2006
DQ647827	ITS	PO	China	<i>Pinus, Betula, Juglans</i>	Mountain	AFTOL-ID 715	Binder & Hibbett 2006
EU078709	ITS	PO	Sweden	<i>Corylus, Betula</i>	Garden lawn	SE03083102	Hedh et al. 2008
EU078710	ITS	PO	Sweden	<i>Betula</i>	Garden lawn	SE03090704	Hedh et al. 2008
EU078711	ITS	PO	Sweden	<i>Betula</i>	Public lawn	SE03091104	Hedh et al. 2008
EU078712	ITS	PO	Sweden	<i>Betula, Salix</i>	Public lawn	SE03091215	Hedh et al. 2008
EU078713	ITS	PO	Sweden	<i>Betula</i>	Garden lawn	SE03092501	Hedh et al. 2008

Accession number	Locus	PS ^a	Country	Recorded trees	Environment type	Herbarium number	Reference
EU078714	ITS	PO	Sweden	<i>Populus, Betula</i>	Wetland, sandfill	PAO03090701	Hedh et al. 2008
EU078715	ITS	PO	Norway	<i>Tilia</i>	Garden lawn	IA04090201	Hedh et al. 2008
EU078716	ITS	PI	Norway	<i>Picea, Betula</i>	Forest	AT04083001	Hedh et al. 2008
EU078717	ITS	PI	Sweden	<i>Betula, Quercus</i>	Forest	SE04071201	Hedh et al. 2008
EU078721	ITS	PI	Canada	<i>Betula, Populus</i>	Forest	CanLH14503	Hedh et al. 2008
EU078737	ITS	PI	CZ	–	–	CBS 477.89	Hedh et al. 2008
EU078739	ITS	PI	Netherlands	–	–	CBS 591.89	Hedh et al. 2008
EU078741	ITS	PA	Sweden	<i>Quercus, Betula</i>	Pasture	SE03100403	Hedh et al. 2008
EU078742	ITS	PC	Sweden	<i>Betula</i>	Public lawn	PAO03090703	Hedh et al. 2008
EU078743	ITS	PC	Sweden	<i>Populus, Corylus</i>	Pasture	SE03100401	Hedh et al. 2008
EU084665	ITS	PO	Germany	<i>Tilia</i>	–	CH 290_98	Hedh et al. 2008
EU084666	ITS	PA	Germany	<i>Tilia</i>	–	CH 243_97	Hedh et al. 2008
EU346878	ITS	PC	Germany	–	–	He1	Unpublished
FR852277	ITS	PC	Iran	–	–	Mycorrhiza clone Ir1	Bahram et al. 2012
GQ389624	ITS	PC	Germany	–	–	FRA	Hryniewicz et al. 2010
JN197698	ITS	PC	Europe	<i>Alnus</i>	–	Mycorrhiza clone I016	Unpublished
JN198064	ITS	PC	Europe	<i>Alnus</i>	–	Mycorrhiza clone S329	Unpublished
JN661711	ITS	PA	England	<i>Mixed forest</i>	–	A.E. Hills 2004209 (K)	Vellinga et al. 2012
JN661712	ITS	PA	England	<i>Quercus</i>	–	A.E. Hills 2005047 (K)	Vellinga et al. 2012
JN661713	ITS	PA	England	<i>Salix</i>	–	A.E. Hills 2005059 (K)	Vellinga et al. 2012
JN661714	ITS	PA	England	<i>Tilia</i>	Park land	A.E. Hills 2005086 (K)	Vellinga et al. 2012
JN661715	ITS	PA	France		–	A.E. Hills 2007039 (K)	Vellinga et al. 2012

Accession number	Locus	PS ^a	Country	Recorded trees	Environment type	Herbarium number	Reference
JN661717	ITS	PA	France	<i>Quercus and Populus</i>	–	A.E. Hills 2007045 (K)	Vellinga et al. 2012
JN661718	ITS	PA	Italy	–	–	IB1997_0980	Vellinga et al. 2012
JN661719	ITS	PA	Italy	<i>Populus</i>	–	M. Contu (UC) s.n.	Vellinga et al. 2012
JN661721	ITS	PIw	England	<i>Betula</i>	–	A.E. Hills 2005067 (K)	Vellinga et al. 2012
JN661722	ITS	PI	USA, TN	<i>Picea</i>	Mountain forest	TENN64458	Vellinga et al. 2012
JN661723	ITS	PI	USA, MI	–	–	M. Kuo 09130703 (UC)	Vellinga et al. 2012
JN661724	ITS	PI	England	–	–	S. Kelly s.n.	Vellinga et al. 2012
JN661725	ITS	PC	USA, CA	<i>Corylus, Quercus</i>	–	D. Deshazer DD535	Vellinga et al. 2012
JN661726	ITS	PC	USA, CA	<i>Betula</i>	–	E.C. Vellinga 3211b (UC)	Vellinga et al. 2012
JN661727	ITS	PC	USA, CA	<i>Betula</i>	–	E.C. Vellinga 3336 (UC)	Vellinga et al. 2012
JN661728	ITS	PC	USA, WA	<i>Betula</i>	Park	E.C. Vellinga s.n. (UC)	Vellinga et al. 2012
JN673368	ITS	PIw	Italy	<i>Betula, Quercus</i>	Forest	TO BP05	Gelardi et al. 2011
JQ283964	ITS	PO	China	–	–	JZBD2100002	Unpublished
JQ436860	<i>gpd</i>	PA	France (Corse)	<i>Quercus, Pinus</i>	Forest	Co09	Moreau et al. 2013
JQ436860	<i>gpd</i>	PO	France (Nord)	<i>Populus</i>	City park	Lille09	Moreau et al. 2013

a

PS = phylogenetic species; PA = *Paxillus ammoniavirescens*; PC = *Paxillus cuprinus*; PI = *Paxillus involutus*; PIw = *Paxillus involutus* white variant; PO = *Paxillus obscurisporus*.

All French voucher specimens are available at the Laboratoire Evolution et Diversité Biologique, Université Paul Sabatier Toulouse 3 and the Italian collection is in the Torino (TO) herbarium.

Isolates and culture conditions

Pure mycelial cultures were obtained from basidiome fragments placed on solid malt extract peptone (MP) medium [50 mg L⁻¹ CaCl₂, 25 mg L⁻¹ NaCl, 500 mg L⁻¹ KH₂PO₄, 250 mg L⁻¹ (NH₄)₂HPO₄, 150 mg L⁻¹ MgSO₄·7H₂O, 1 mg L⁻¹ FeCl₃·6H₂O, 1 g L⁻¹ casein peptone

(ThermoFisher, Illkirch, France), 5 g L⁻¹ malt extract (Merck, Darmstadt, Germany), 5 g L⁻¹ glucose (Merck, Darmstadt, Germany), 0.1 mg L⁻¹ thiamin (ACROS-ThermoFisher, Illkirch, France), 0.1 mg L⁻¹ ZnSO₄·7H₂O, 0.1 mg L⁻¹ H₃BO₃, 10 µg L⁻¹ MnSO₄·7H₂O, 3 µg L⁻¹ CuSO₄·7H₂O, 3 µg L⁻¹ AlCl₃, 3 µg L⁻¹ NiCl₂·6H₂O, 3 µg L⁻¹ MoNa₂, 1 µg L⁻¹ KI, 10–15 g L⁻¹ Agar (Merck, Darmstadt, Germany)] supplemented with 100 mg L⁻¹ Na-ampicillin, 120 mg L⁻¹ SO₄-streptomycin, 15 mg L⁻¹ tetracycline, 30 mg L⁻¹ chloramphenicol, 30 mg L⁻¹ SO₄-kanamycin, and 100 µl L⁻¹ of thiabendazole lactate from a 23 % stock solution. Plates were incubated at 24 °C in the dark.

Mycelia were cultured 35 d on complete solid MP medium and on poor MP medium containing only 2.5 g L⁻¹ glucose as the carbon source.

All cultures are available at the Laboratoire Evolution et Diversité Biologique, Université Paul Sabatier Toulouse 3.

Morphological observations

Freshly collected basidiomes were observed and the reaction of their pileus surface to a 50 % ammonia solution was tested. Spore prints were obtained and colours of fresh spore prints with sufficient spore deposits were coded according to [Kornerup & Wanscher \(1967\)](#). As much as possible, comparisons of spore prints of the different species were done at the same time.

Microscopic observations were made using a Zeiss AxioLab A1 (Jena, Germany) light microscope (objectives 10×, 40×, and 100× oil immersion) and all measurements were performed using an AxioCam Erc 5s camera with integrated software. Spore shape was defined according to the glossary of the Flora Agaricina Neerlandica ([Vellinga 1988](#)). All spore measurements were carried out on spores collected from spore prints and mounted in water. Selected quantitative spore characters including spore length (apiculus excluded), spore width, and the *Q* ratio between spore length and spore width were analysed in order to assess whether they were statistically different among *Paxillus* species and to evaluate their variation within each species. Spore sets of 289, 239, 99, and 430 spores yielded by the spore prints of six, five, two, and eight specimens of *Paxillus cuprinus*, *Paxillus involutus*, *Paxillus obscurisporus*, and *Paxillus ammoniavirescens* respectively ([Table 1](#)) were analysed. Univariate statistics were performed to determine the mean, median, and variance values of the measurements. ANOVA tests and *post hoc* pairwise Tukey's tests were used to compare each spore parameter between the four species. Within each species, variations among specimens were also tested by ANOVA and *post hoc* pairwise Tukey's test. To meet the assumptions of ANOVA, data were transformed using Box–Cox transformations. All statistical tests were done with PAST software ([Hammer et al. 2001](#)).

The other microscopic elements were observed from dried preserved specimens, rehydrated in ammoniacal Congo red (1 % Congo red in 12.5 % ammonia solution) or in 5 % KOH for cystidia.

Molecular methods

Eighty-two basidiomes from the 47 collections were used for phylogenetic analyses ([Table 1](#)) and *Paxillus rubicundulus* Ve08.2h10, whose complete genome is sequenced (<http://genome.jgi-psf.org/Paxru1/Paxru1.home.html>), was used as an outgroup. Fungal DNA was extracted from dried basidiomes or from fragments of basidiomes stored at –20 °C in Nuclei Lysis Solution (Promega, Charbonnières-les-Bains, France), using the Wizard Genomic DNA Purification kit (Promega, Charbonnières-les-Bains, France). The final pellet was resuspended in 40 µl of sterile ultra high quality (UHQ) water.

The internal transcribed spacer (ITS) rDNA, *tef1- α* , and *gpd* nuclear regions were PCR-amplified as in [Jargeat et al. \(2010\)](#).

The PCR products were sequenced by GENOSCREEN (Lille, France) or MilleGen (Labège, France) with the same primers as for PCR amplifications. The sequences are available from the GenBank database under accession numbers KF261354–KF261553.

The ITS data set was supplemented with 58 *Paxillus* sequences from GenBank, mainly generated by [Hedh et al. \(2008\)](#) and [Vellinga et al. \(2012\)](#) ([Table 2](#)). They include the ITS sequences of white variants ([JN661721](#) and [JN673368](#)), the ITS sequence of *Paxillus ammoniavirescens* (IB1997_0980) and *Paxillus obscurisporus* (CH290_98) types, the ITS sequences of the genome-sequenced strain ATCC 200175, and some sequences obtained from ectomycorrhizal tips. One *tef* and two *gpd* *Paxillus* sequences from GenBank were also included in the analyses ([Table 2](#)).

All our references to species from the literature refer to collections that are recognized by sequence data.

Phylogenetic and statistical analyses

Sequence chromatograms were manually checked to identify heterozygous nucleotide position and sequences were manually corrected using BioEdit software ([Hall 1999](#)). Multiple sequence alignments were conducted for the three nuclear regions ITS rDNA, *gpd*, and *tef* in SeaView v4.0 ([Galtier et al. 1996](#)) using MUSCLE v3.7 ([Edgar 2004](#)). The three alignments were concatenated into a single sequence alignment of 1686 bp.

Alignments are available on [treebase.org](#) under the following link <http://purl.org/phylo/treebase/phyloids/study/TB2:S14414>.

The evolutionary model was selected by using jModelTest-2.1.4 ([Darriba et al. 2012](#)). Phylogenetic analyses were performed using the Bayesian inference with MrBayes v3.2 ([Ronquist et al. 2012](#)), with six Markov Chain Monte Carlo (MCMC) ran simultaneously for 2 000 000 generations (*tef* and *gpd*) or 3 000 000 generations (ITS, concatenated) under GTR + gamma evolutionary model. Trees were saved every 500 generations and the first 25 % were discarded as ‘burn-in’. For the remaining trees, a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities. Phylogenetic trees were visualized and edited with FigTree v1.4.0 available at <http://tree.bio.ed.ac.uk/software/figtree/>.

The topological congruence between the trees obtained from the three loci was tested by the maximum agreement subtree (MAST) method using the online calculator of congruence index I_{cong} ([De Vienne et al. 2007](#)) available at <http://max2.es.e.u-psud.fr/icong/index.help.html>.

In order to use the information in the heterozygous nucleotide position and thus calculate more accurate estimates of molecular ITS variations among all samples (our collections and sequences from GenBank), a haplotype data set was constructed with homozygous sequences and sequences for which heterozygosity could be resolved without cloning, *i.e.* with only one heterozygous position. For example, in a DNA sequence (‘genotype’) containing an ‘R’ (=A/G), the two resulting sequence haplotypes will include either an ‘A’ or a ‘G’. Sequences with more than one heterozygous position were not taken into account. Estimates of molecular variation [nucleotide diversity (π) and nucleotide divergence (K) calculated using Juke–Cantor distance] were calculated in DnaSP 5.10.01 ([Librado & Rozas 2009](#)). An estimation of genetic diversity was also obtained by analysing the phylogenetically informative positions as well as private mutations (mutations present

in only one sequence of the overall data set) with DIVEIN ([Deng et al. 2010](http://indra.mullins.microbiol.washington.edu/DIVEIN/)) <http://indra.mullins.microbiol.washington.edu/DIVEIN/>.

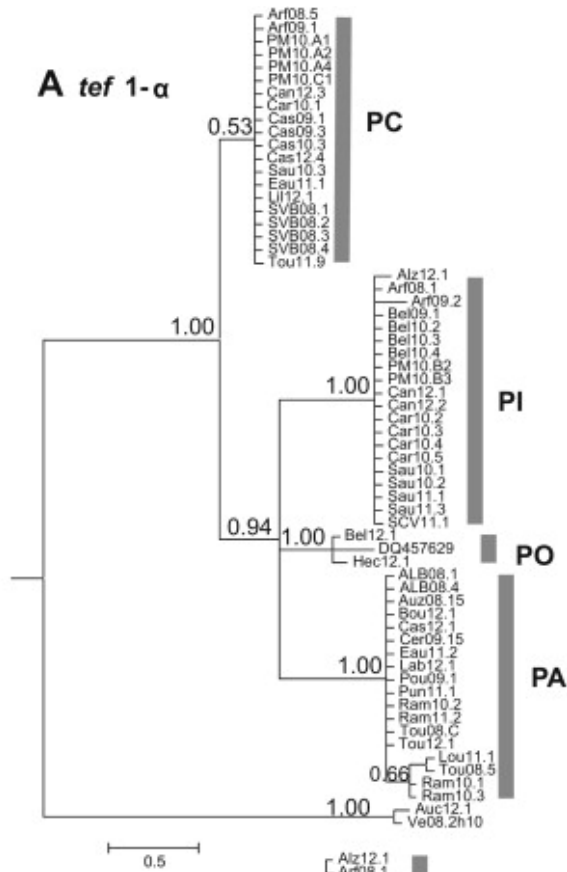
Results

Phylogenetic analyses and genetic diversity

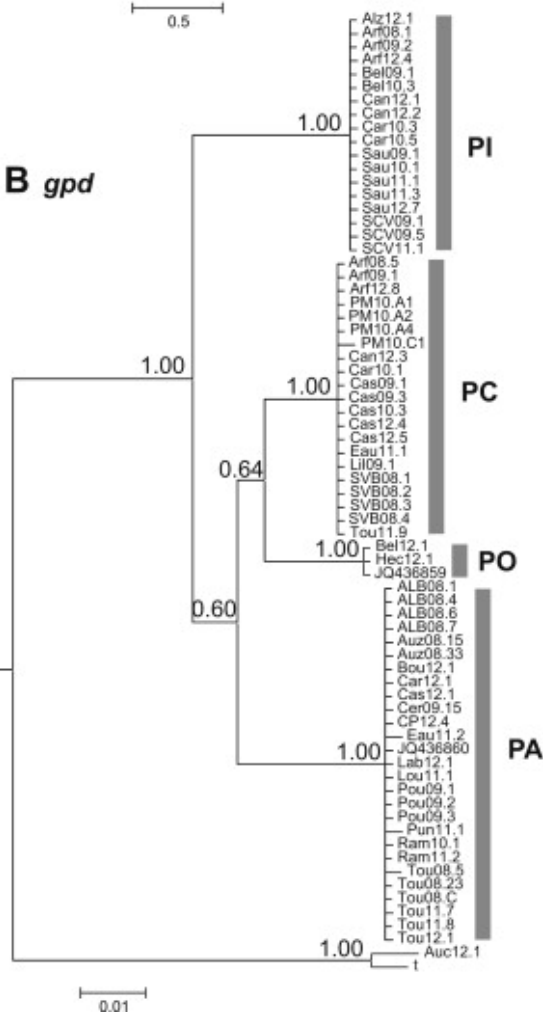
Partial 507-bp ITS sequences were generated from 69 samples, 522-bp *tef1*- α sequences from 62 samples, and 646-bp *gpd* sequences from 69 samples, all including two *Paxillus rubicundulus* samples. The ITS, *tef*, and *gpd* phylogenies support four clades in the *Paxillus involutus* complex and *P. rubicundulus* is placed as an outgroup ([Figs 1](#) and [2](#)). According to type sequences, two of these phylogenetic species were assigned to *Paxillus ammoniavirescens* and *Paxillus obscurisporus*. A third branch, including the genome-sequenced strain ATCC 200175 ([AY585913](#)), *P. involutus* f. *eburneus* ([JN673368](#)), and an English whitish collection ([JN661721](#)), represented *P. involutus*. The fourth phylospecies is called *Paxillus cuprinus* hereafter.

ammoniavirescens, PC = *P. cuprinus*, PI = *P. involutus*, PO = *P. obscurisporus*. All strains and gene accession numbers are presented in [Tables 1](#) and [2](#).

A *tef 1-α*



B *gpd*



C Concatenated

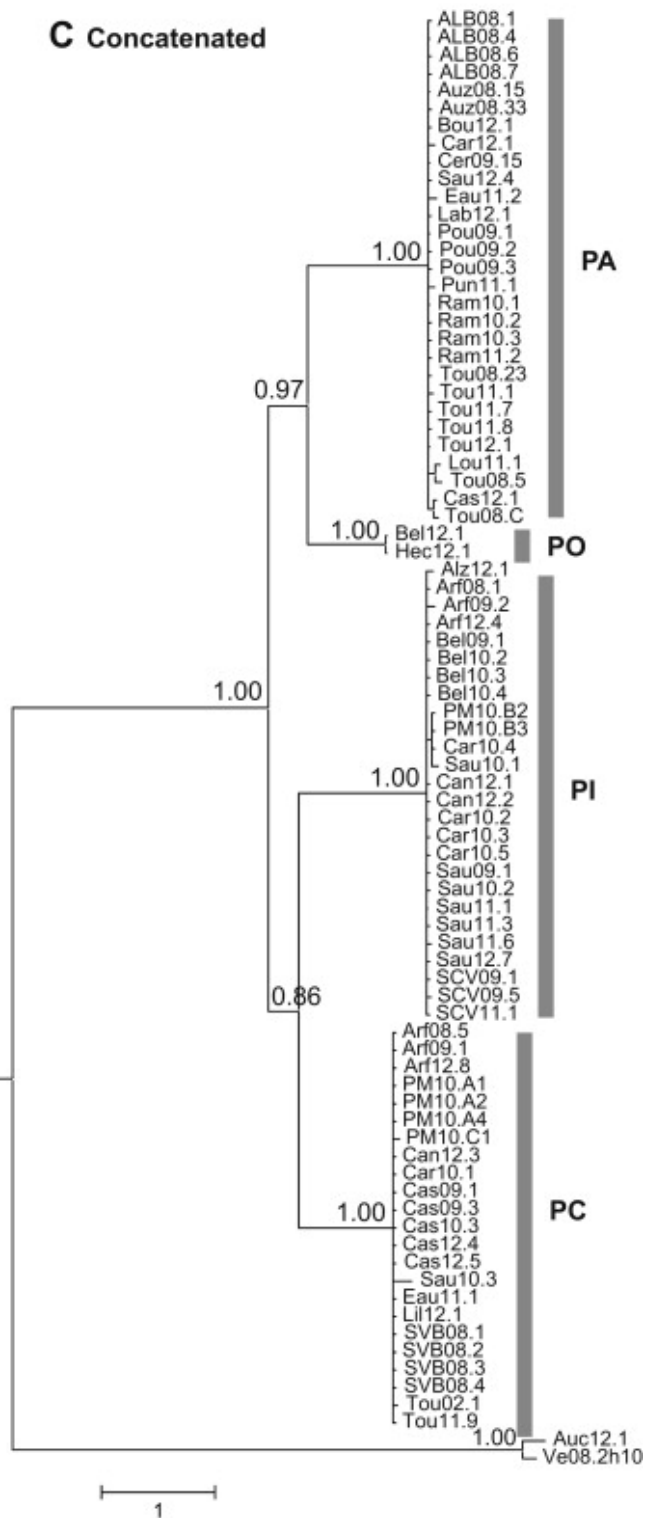


Fig 2.

Phylogenies of *Paxillus involutus* and allies derived from the *tef1*- α (A), *gpd* (B), and concatenated sequences (C) using Bayesian analyses. The Bayesian PPs above 0.5 are shown above branches. The tree was rooted using sequences from *P. rubicundulus*. The scale shows the expected number of changes per nucleotide. Four different phylogenetically species are identified: PA = *P. ammoniavirescens*, PC = *P. cuprinus*, PI = *P. involutus*, PO = *P. obscurisporus*. All strains and gene accession numbers are presented in [Tables 1](#) and [2](#).

In the ITS tree ([Fig 1](#)), *P. cuprinus* and *P. involutus* on the one hand and *P. ammoniavirescens* and *P. obscurisporus* on the other hand are sister-groups. The *tef* tree ([Fig 2A](#)) reveals four well-supported branches where *P. ammoniavirescens*, *P. obscurisporus*, and *P. involutus* are clustered while *P. cuprinus* is more distant. In the *gpd* tree ([Fig 2B](#)), *P. involutus* is the most distant while *P. cuprinus* and *P. obscurisporus* are related. However, nodes are weakly supported and congruence of the three trees was confirmed ($I_{\text{cong}} = 2.95\text{--}3$, P value = $2.45 \text{ E}^{-17}\text{--}9.47 \text{ E}^{-16}$). The tree obtained with the three concatenated genes ([Fig 2C](#)) is similar to the ITS tree. Each specimen was consistently assigned to the same phylogenetic species.

As most of the ITS sequences contained only one heterozygous position, it was possible to generate a haplophase data set, with sequences from this study and from GenBank. The numbers of ITS haplotypes were four for *P. cuprinus*, seven for *P. involutus*, nine for *P. ammoniavirescens*, and five for *P. obscurisporus*. ITS nucleotide diversity (π), which represents sequence variation, ranged between 0.008 (PA–PO) and 0.023 (PA–PC) at the intergroup level but ranged between 0.001 (*P. cuprinus*) and 0.003 (*P. ammoniavirescens*) at the intragroup level ([Table 3](#)).

Table 3.

Statistical data calculated at the intra and interspecific levels for the four phylogenetic species (PA = *P. ammoniavirescens*; PC = *P. cuprinus*; PI = *P. involutus*; PO = *P. obscurisporus*) with the ITS haplophase data set.

	Nb of studied location (this study + literature)	Number of sequences (this study + literature)	Nucleotide diversity π	Nucleotide divergence K (%)
<i>P. ammoniavirescens</i>	13 + 13	20 + 11	0.003	0.26
<i>P. involutus</i>	8 + 10	32 + 10	0.002	0.2
<i>P. cuprinus</i>	11 + 12	25 + 14	0.001	0.12
<i>P. obscurisporus</i>	2 + 17	3 + 15	0.002	0.15
PA–PI		72	0.018	3.7
PA–PC		69	0.023	4.7
PA–PO		48	0.008	1.6
PI–PC		81	0.013	2.5
PI–PO		60	0.013	3
PC–PO		57	0.015	3.5

Genetic diversity analysis of the ITS data set ([Table 3](#)) indicated that *P. cuprinus* ITS sequences were more similar to *P. involutus* ITS sequences ($K = 2.5$ %) and *P. ammoniavirescens* ITS

sequences were highly similar to *P. obscurisporus* ITS sequences ($K = 1.6\%$). When we compared more distant species (PA–PI, PA–PC, PI–PO, PC–PO), nucleotide divergence K was always higher than 3% . At the intraspecific level, nucleotide divergence ranged between 0.12% (*P. cuprinus*) and 0.26% (*P. ammoniavirescens*).

The *gpd* and *tef* fragments contained many heterozygous positions (2–5) and it was not possible to deduce the different alleles. However the number of polymorphic informative sites and the total number of variable sites allowed us to estimate genetic diversity. The lowest variability was obtained for *P. cuprinus* (mean values of 2.67 and 4.67) and the highest variability was observed for *P. ammoniavirescens* (mean values of 8.67 and 14.00) (Table 4).

Table 4.

Number of polymorphic informative sites and total number of variable sites (including indels and single variations).

	Number of sequences ^a			Number of polymorphic informative sites				Total number of variable sites			
	ITS	<i>tef</i>	<i>gpd</i>	ITS	<i>tef</i>	<i>gpd</i>	Mean	ITS	<i>tef</i>	<i>gpd</i>	Mean
<i>P. ammoniavirescens</i>	32	18	27	5	7	14	8.67	11	8	23	14.00
<i>P. involutus</i>	35	20	17	3	4	2	3.00	5	7	9	7.00
<i>P. cuprinus</i>	34	20	22	1	3	4	2.67	4	3	7	4.67
<i>P. obscurisporus</i>	19	3	3	2	nd	nd	nd	4	6	7	5.67

nd: not determined.

a

Including GenBank sequences.

Macroscopic features and chemical reactions

Once all the specimens were assigned to a phyllospecies, taxonomic characters were examined. Based on field observations of fresh specimens, general aspect distinguished two groups: *Paxillus obscurisporus* and in some cases *Paxillus ammoniavirescens* (Tou08, Tou11.A, Tou12, Auz08, Pou09, Ram10 collections) had robust basidiomes, a short stipe and grew in dense clusters whereas some other *P. ammoniavirescens* collections (Cer09, Eau11, ALB08, Sau12.R), *Paxillus involutus* and *Paxillus cuprinus* were characterized by less massive basidiomes with a more slender stipe, growing in groups of separated basidiomes or up to three connate basidiomes (Fig 3B–D). More individual characters revealed that *P. cuprinus*, *P. involutus*, and *P. obscurisporus* displayed duller colours than *P. ammoniavirescens*. This species typically has a yellow shade on the whole basidiome and its pileus is often brightly coloured, yellow–brown to orange–brown or fulvous. *Paxillus cuprinus* had an olivaceous shade when young and then turned more brownish and finally coppery to reddish brown (Fig 3A–C). This colour change was particularly intense in dry weather (Fig 3B). At first, *P. obscurisporus* was lead-grey to ochre–grey and sometimes it also turned coppery with age. *Paxillus involutus* had a broader range of colours, mostly ochraceous brown, grey–brown or rusty brown, more rarely with a conspicuous olivaceous shade. All taxa had a more or less enrolled margin (Fig 3D and E for *P. cuprinus*). In contrast, *P. involutus*, especially young

specimens, was the only one that frequently had a broad umbonate pileus. At maturity, outwards adpressed radiating fibrillose squamules sometimes occurred on the pileus of the four species, although more rarely in *P. involutus*. The viscosity of the pileus surface was especially important in *P. ammoniavirescens* which was even glutinous in a few cases. The other three taxa were moderately slimy in wet weather. A fleeting green reaction to ammonia was observed for the pileus surface of *P. ammoniavirescens* basidiomes, especially when the pileus was slimy. For the other three species, the reaction yielded a reddish brown to dark grey colour.

Fig 3.



Various aspects of *Paxillus cuprinus* basidiomes collected in France. (A) Holotype Cas10.3 from Castanet-Tolosan, (Haute-Garonne), 2010; (B) Cas12 collection from Castanet-Tolosan, (Haute-Garonne), 2012; (C) Mature basidiomes from PM10.A collection from Prats-de-Mollo, (Pyrénées-Orientales), 2010; (D) Young basidiomes from collection Tou11 from Toulouse, (Haute-Garonne), 2011; (E and F) Young basidiome from collection Eau11

from Eauzes, (Gers), 2011. The small young specimen is *Gyrodon lividus*. Scale bars = 2 cm.

The flesh of the four species was yellowish at first and then turned brown or rusty brown. However, the speed and the intensity of this change varied depending on the collection and the age of the specimens (it was more obvious with young specimens). In *P. cuprinus*, the flesh immediately and intensively changed to reddish brown after cutting or wounding (see the margin of the pileus and gills of the young specimen, [Fig 3D](#)).

Spore prints of *P. ammoniavirescens* were usually ochraceous brown, often clearly greenish tinged (5D6–5E7). *Paxillus cuprinus* spore deposits usually displayed much darker shades, more reddish and without a distinctly greenish tinge (5E7–6E6–6E7). *Paxillus involutus* spore prints had an intermediate colour range (5E6–5E7–6E6) but the colour was mostly much more reddish compared with *P. ammoniavirescens*. Lastly, *P. obscurisporus* was characterized by deep reddish brown spore prints (7E6–7E7 fading to 5E6–5E7 over time). Whatever the species, spore print colour gradually changed during storage and turned to ochraceous brown (5D6 or 5E7).

Microscopic features and spore size statistical analysis

Twenty-one spore prints were examined to compare spore morphology across species. *Paxillus cuprinus* spores were typically ovoid or amygdaloid with a frequent slight depression on their dorsal side near the apex and therefore showed a slight apical constriction ([Fig 4A](#)). *Paxillus involutus* spores were generally ovoid–amygdaliform, with sometimes the same dorsal depression as *P. cuprinus* spores. *Paxillus ammoniavirescens* spore shapes varied widely depending on the specimens. They were recorded as broadly ellipsoid as in isolate Eau11.2 or almost oblong (isolate Cas12.1). A few spores were found rather amygdaliform (Car12.1) or, as in isolate Pun11.1, some spores were subphaseoliform in side view and others displayed a supraapical depression. *Paxillus obscurisporus* exhibited broadly elliptical spores with an occasional amygdaliform trend, especially in the largest spores. Spore length values were significantly higher for *P. cuprinus* than for the other three species ([Table 5](#); [Fig 5](#)). However, *P. involutus* specimen SCV11.1 and *P. ammoniavirescens* Cas12.1 also had relatively long spores and overlapped with *P. cuprinus* ([Fig 5](#)). No significant difference in spore width was found between *P. cuprinus* and *P. obscurisporus* ([Table 5](#); [Fig 5](#)). The spores of both these species were significantly wider than *P. involutus* spores, while *P. ammoniavirescens* spores were the narrowest ([Table 5](#); [Fig 5](#)), even if the differences were tenuous. There was no significant difference in the spore length/spore width *Q* ratios of *P. cuprinus*, *P. involutus*, and *P. ammoniavirescens* but the *Q* ratio of *P. obscurisporus* spores was significantly lower ([Table 5](#); [Fig 5](#)). The lowest variance for the three parameters was always found for *P. cuprinus* ([Fig 5](#)).

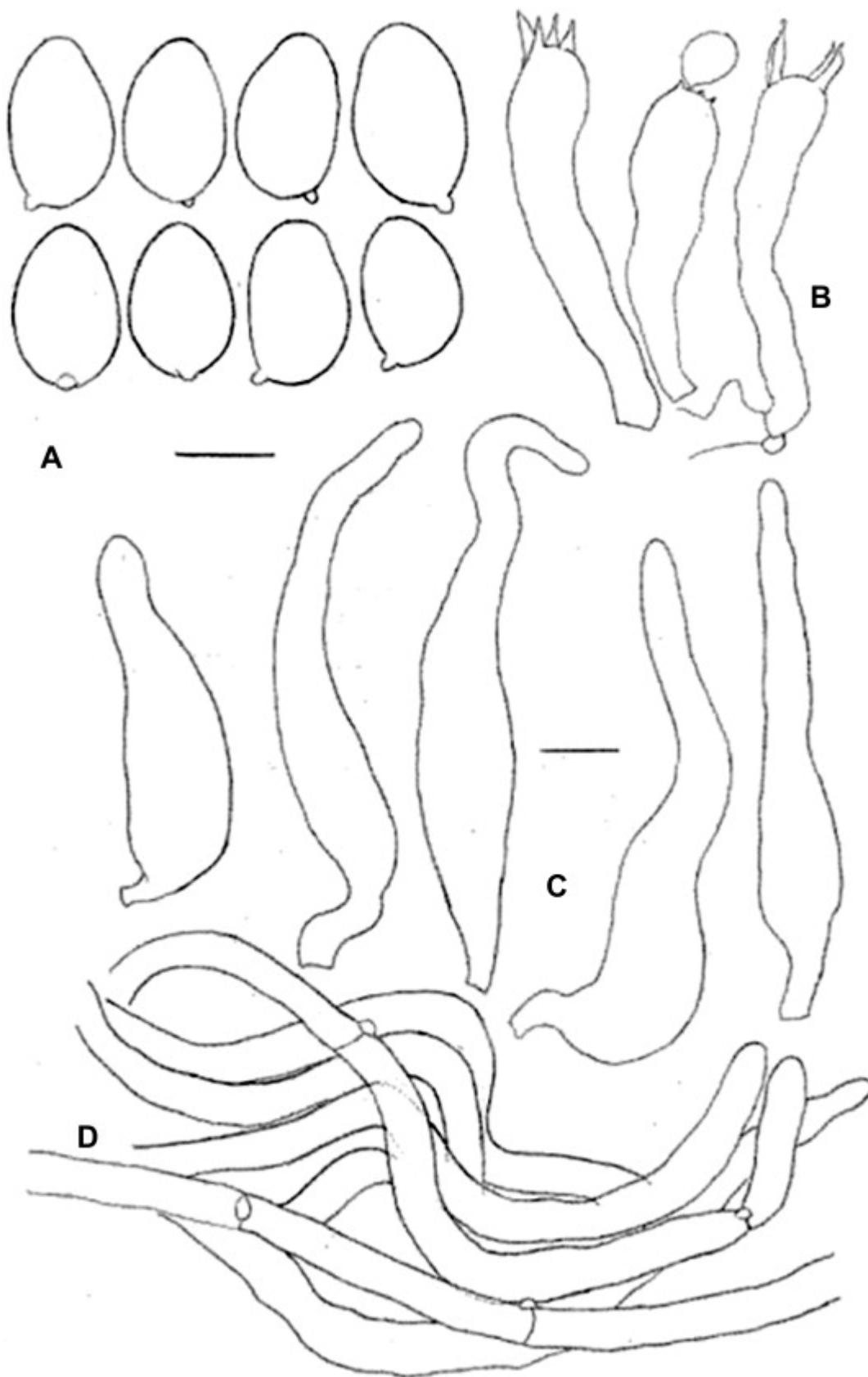


Fig 4.

Microscopic features of *Paxillus cuprinus* observed on the holotype specimen Cas10.3. (A) Basidiospores; (B) Basidia; (C) Pleurocystidia; (D) Pileipellis (cutis). Scale bar = 5 μ m.

Table 5.

Sizes of microscopical elements.

	Spore size (µm)	Spore length/width <i>Q</i> ratio	Cystidia size (µm)	Hyphae diameter (µm)
<i>P. cuprinus</i>	(7.0)7.4– 9.4(11.5) × (4.5)4.9– 5.7(6.2) <i>n</i> = 289	(1.30)1.41– 1.75(2.13) <i>n</i> = 289	(47)51– 123(137) × (7)8– 16(19) <i>n</i> = 73	(4.0)4.6– 7.7(8.1) <i>n</i> = 70
<i>P. involutus</i>	(6.4)6.9– 9.2(10.6) × (4.3)4.5– 5.6(6.0) <i>n</i> = 239	(1.32)1.39– 1.80 (1.98) <i>n</i> = 239	(43)48–86 (88) × (8)9–14(15) <i>n</i> = 48	(5.1)5.2– 8.2(8.4) <i>n</i> = 68
<i>P. ammoniavirescens</i>	(6.3)6.6– 9.4(11.6) × (4.0)4.4– 5.4(6.3) <i>n</i> = 430	(1.30)1.40– 1.80 (1.96) <i>n</i> = 430	(41)50–95 (108) × (6)9– 17(19) <i>n</i> = 51	(4.6)5.2– 8.8(9.7) <i>n</i> = 58
<i>P. obscurisporus</i>	(6.5)6.9– 8.9(10.2) × (4.8)5.0– 6.2(6.5) <i>n</i> = 99	(1.24)1.31– 1.68 (1.90) <i>n</i> = 99	(37)51–91 (99) × (7)10– 19(21) <i>n</i> = 56	(4.9)5.2– 8.2(8.9) <i>n</i> = 56

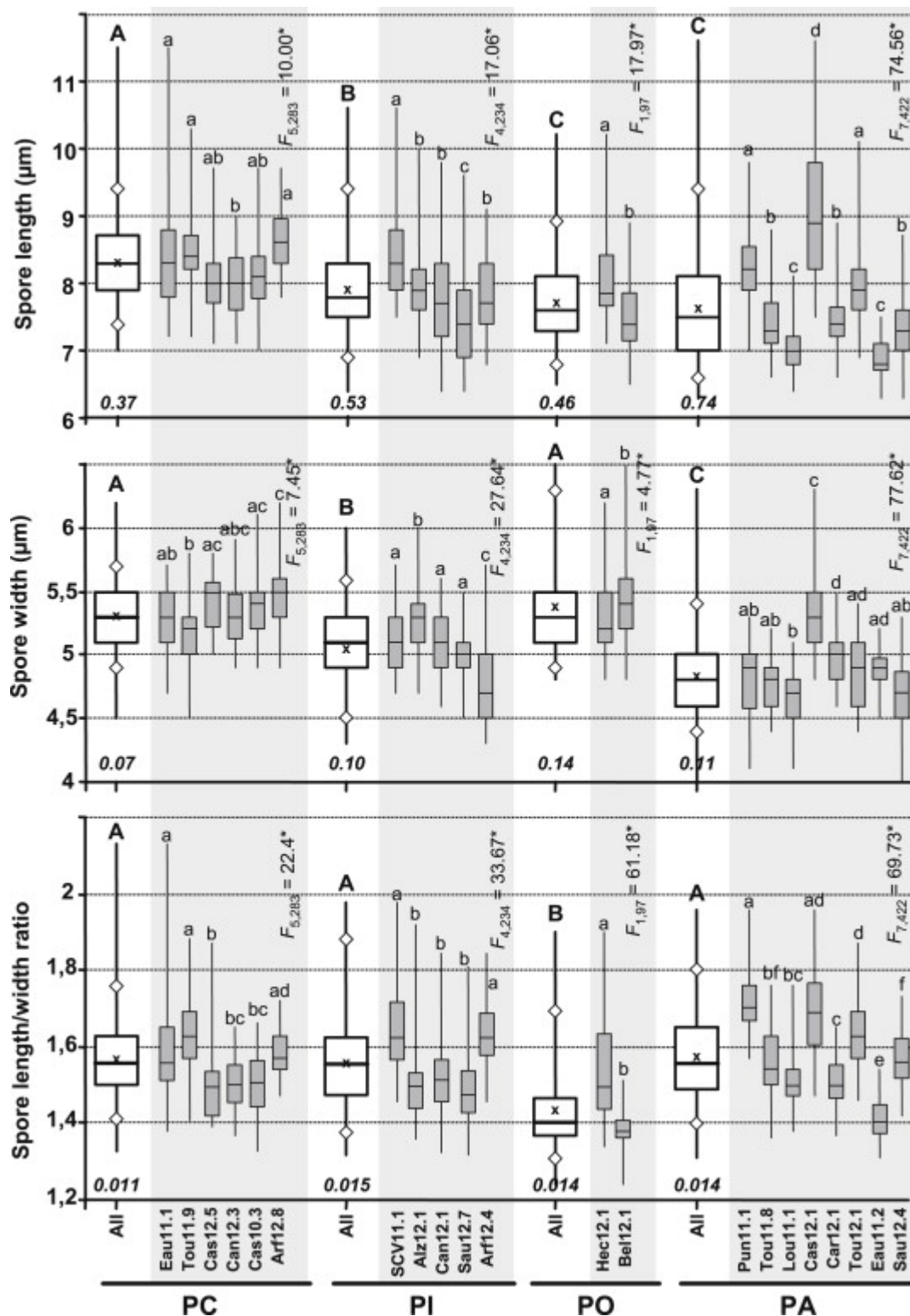


Fig 5.

Comparison of three spore parameters (length; width, and length-over-width ratio) among the four species *P. cuprinus* (PC), *P. involutus* (PI), *P. obscurisporus* (PO), and *P. ammoniavirescens* (PA). Large white boxplots correspond to data compilation at the species level. Each box contains 50 % of the data (interquartile range). White diamonds (large boxplots) indicate the 5th and 95th percentiles and vertical lines delineate the range of values. Horizontal lines and x symbols within boxes correspond to the median and mean values respectively. Bold italic numbers indicate variance of each parameter within each

species. Capital letters indicate significant differences ($P < 0.05$) between species for that particular parameter (ANOVA and Tukey's *post hoc* test). Small grey boxplots over shaded areas detail values recorded for each specimen of each species. For each parameter and each species, results of 'among specimens' comparisons are obtained from the F values provided by ANOVA analyses (*, $P < 0.0001$). Small letters indicate differences ($P < 0.05$) among specimens within each species as determined through Tukey's *post hoc* tests following ANOVA. Box–Cox-transformed data were used to perform all ANOVA analyses whereas untransformed values are presented in the figure.

Significant variations of each parameter were recorded across individuals of each species ($P < 0.0001$ for all F values of ANOVA; [Fig 5](#)) underlining interindividual differences within each species. Whatever the parameter, the highest interindividual variation estimated with F values from ANOVA analysis was found for *P. ammoniavirescens* ([Fig 5](#)). The lowest spore length variations ($F_{5,283} = 10.00$; [Fig 5](#)) and Q ratio ($F_{5,283} = 22.4$; [Fig 5](#)) were found among *P. cuprinus* individuals and the lowest spore width variations among *P. obscurisporus* individuals ($F_{1,97} = 4.77$; [Fig 5](#)), but only two specimens were studied for this species. Spore width variation among *P. cuprinus* individuals was also low ($F_{5,283} = 7.45$). For each spore parameter, the highest number of significant differences was recorded when comparing *P. ammoniavirescens* individuals together whereas the lowest number was observed among *P. cuprinus* individuals (from pairwise *post hoc* Tukey's test, [Fig 5](#)). Along with F values, this result suggests that *P. cuprinus* exhibited the lowest interindividual variability in spore parameters and *P. ammoniavirescens* the highest.

Hymenial cystidia were found in all four species. Cheilo and pleurocystidia were very much alike and their sizes were grouped together. The smallest cystidia were observed in *P. involutus* ([Table 5](#)). They were fairly short and broad in *P. ammoniavirescens* and slightly larger in *P. cuprinus* and *P. obscurisporus* ([Table 5](#)). The pileipellis is a cutis to an intricate trichoderm and hypha diameter was similar in all four species ([Table 5](#)). A gelatinous matrix was occasionally observed in all four species but the pileipellis was usually clearly gelatinized (ixocutis to ixotrichoderm) in *P. ammoniavirescens*, in relation to the viscosity of the pileus surface. The superficial hyphae of the rhizomorphs were finely dotted by microcrystals in *P. cuprinus*, *P. involutus*, and *P. obscurisporus* and incrustated by crystals up to $2.5\ \mu\text{m}$ in *P. ammoniavirescens*.

Cultural features

Pure mycelial cultures were obtained from fresh basidiomes of *Paxillus cuprinus* (13 isolates), *Paxillus involutus* (ten isolates), and *Paxillus ammoniavirescens* (32 isolates out of which 19 were studied). Four to five plates per isolate were examined and mycelium diameter was measured. *Paxillus cuprinus* mycelia ([Fig 6](#)) were aerial grey–beige, with brown droplets. They had a limited growth ($3.16\ \text{cm} \pm 0.77$ in diameter, $n = 60$) that stopped after 1 m of incubation. Mycelia cultivated on rich medium containing high carbon quantities (glucose, malt extract, casein) were more compact and generated a brown staining of the medium due to a high production of pigments. *Paxillus involutus* cultures ([Fig 6](#)) were characterized by a limited growth ($3.71\ \text{cm} \pm 0.93$, $n = 40$), pigment production on rich medium, and by the absence of brown droplets. On the contrary, most of the *P. ammoniavirescens* isolates grew quickly, covered the entire medium within 3–4 weeks (average diameter = $4.61\ \text{cm} \pm 1.09$, $n = 81$), with thick aerial mycelium like cotton wool, brown droplets, and sclerotia, on rich medium as well as on poor medium ([Fig 6](#)).

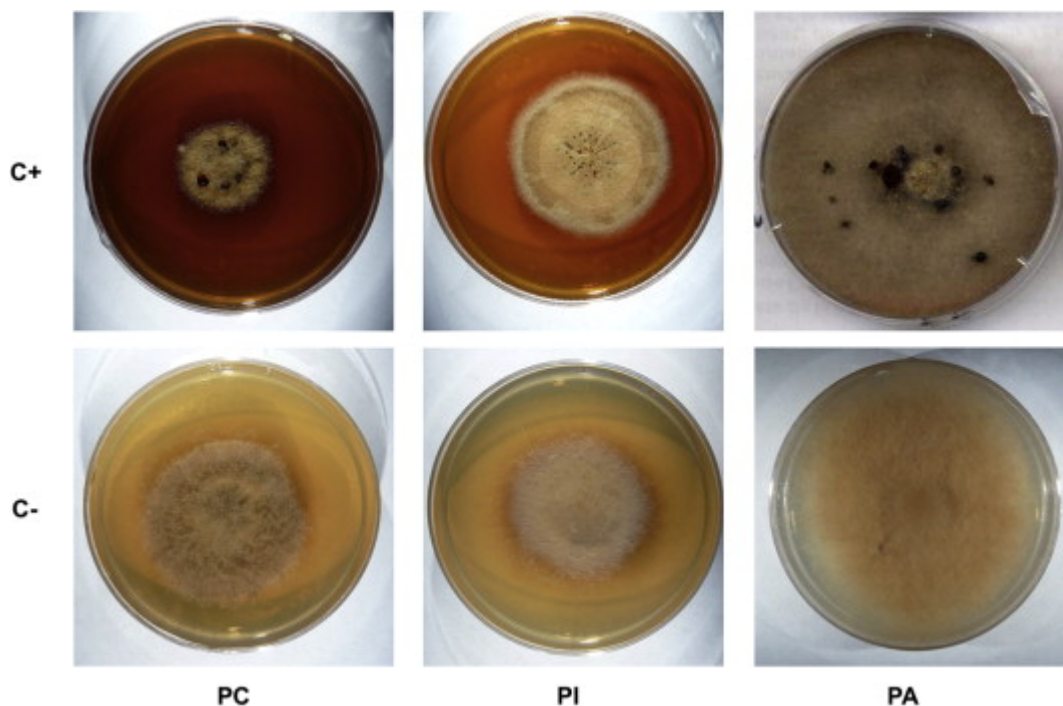


Fig 6.

Mycelia of *Paxillus cuprinus* (PC), *P. involutus* (PI), and *P. ammoniavirescens* (PA) cultivated 35 d on complete MP medium (C+) and on poor MP medium (C-). When cultivated on C+ medium, the three species produce brown pigments diffusing in the substrat and beading on the mycelium. *Paxillus ammoniavirescens* has the highest growth rate on both media.

Ecological preferences and geographic distribution

Ecological preferences of each species were examined through environmental conditions recorded when the specimens were collected ([Table 1](#)) and environmental data provided in the literature ([Table 2](#)).

Sixteen of our *Paxillus cuprinus* collections were found over a large geographic scale in France ([Table 1](#)): in southern France (Ariège, Haute-Garonne, Gers, Hautes-Pyrénées, Pyrénées-Orientales, and Tarn) as well as in northern (Nord) and in eastern (Saône-et-Loire) France. One collection (Vez12) came from North Italy (Emilia Romagna). Literature data indicate that *P. cuprinus* is also present in Germany, Sweden, North America, and Iran ([Table 2](#)). All our collections were found in the vicinity of *Betulaceae* (*Betula*, *Alnus*, *Corylus* or *Ostrya*) ([Table 1](#)) and it was also the case for specimens or mycorrhizal tips recorded from the literature ([Table 2](#)). Basidiomes of *P. cuprinus* were associated to planted *Betula* in urban areas such as city parks or public lawns or with spontaneous *Betulaceae* in open natural environments such as roadsides in forests and river banks ([Table 1](#)) or pastures and unmanaged lawns ([Table 2](#)). Our 14 collections of *Paxillus involutus* were found in eight locations in the south-west of France, more than 400 m above sea level ([Table 1](#)), but *P. involutus* is also present in the north of Europe (Great Britain, the Netherlands, Norway, and Sweden) and in North America (Canada and the USA) ([Table 2](#)). It was found associated to coniferous trees (*Picea*, *Abies*, and *Pinus*) and deciduous trees (*Betula*, *Quercus*, and *Fagus*) but always in forests ([Tables 1](#) and [2](#)).

The 17 *Paxillus ammoniavirescens* collections came from 13 locations in the south of France ([Table 1](#)) and it was also recorded in Sweden, Germany, England, and Italy ([Table 2](#)). It was

collected mainly under deciduous trees (*Populus*, *Betula*, *Salix*, and *Quercus*) but also under coniferous trees (*Cedrus* and *Pinus*). It usually grows in open environments ([Table 1](#)), in urban areas, and in more natural environments (forest edges, forest roadsides, and river banks).

We found only two collections of *Paxillus obscurisporus* in the south–west of France ([Table 1](#)), in mountain locations (630 and 1050 m altitudes) but it is clearly present in the north of Europe (North of France, Sweden, Norway, and Germany) and it has also been reported in China, from mycorrhizal tips ([DQ647827](#), [DQ657629](#), [JQ283964](#)) ([Table 2](#)). Our collections ([Table 1](#)) and specimens from the literature ([Table 2](#)) were found associated to deciduous trees (*Betula*, *Corylus*, *Tilia*, *Populus*, and *Quercus*) in open natural (forest edge) or urban (garden lawn, city park) areas.

The species do not exclude each other. Thus, *P. cuprinus* can be found in the same location as the other species. In Eauze, Toulouse, and Castanet-Tolosan, it was found in the vicinity of *P. ammoniavirescens* and in the forests of Prats-de-Mollo, Saurat, Cardeilhac, Cantaous, and Arfons, not far from *P. involutus*.

Taxonomy

Paxillus involutus (Batsch: Fr.) Fr., *Epicrasis Systematis Mycologici***317** (1838) [MB#191172].

Synonyms: *Agaricus contiguus* Bull., *Herbier de la France***5**: t. 240 (1785) [MB#201927].

Agaricus involutus Batsch, *Elenchus fungorum. Continuatio prima***39** t. 13: 61 (1786) [MB#229635].

Agaricus involutus Batsch: Fr., *Systema mycologicum***1**: 271 (1821).

Omphalia involuta (Batsch) Gray, *A natural arrangement of British plants* **1**: 611 (1821) [MB#486483].

Rhymovis involuta (Batsch) Rabenh., *Deutschlands Kryptogamenflora***1**: 453 (1844) [MB#508583]

Typus: **Germany**, illustration in *Elenchus fungorum – Continuatio prima* (Batsch 1786: Table XIII, Fig 61a–e, iconotype, *lectotypus hic designatus*).
http://www.librifungorum.org/Image.asp?ItemID=2&ImageFileName=IMG_5529.jpg.

Batsch's illustration does not really fit the actual concept of *P. involutus* (Hahn & Agerer 1999) and perhaps depicts *Paxillus obscurisporus* as in the protologue Batsch explained that the specimens are especially large and thick. In any case, we chose here collection Can12.1_B121140 as an epitype in order to avoid more disturbances in this species complex.

Epitype: **France**. Hautes-Pyrénées (65), Cantaous, alt. approx. 590 m, in group of separate basidiomes under *Picea abies* in forest, leg J-P Chaumeton 17 November 2012, Can12.1_B121140. TL127-0001.

Pileus: 4–9(12) cm diam. Convex then expanding, deeply depressed in mature specimen, with broad umbo in central depression, more or less viscid when wet, sticky by drying, with inrolled margin, felted and faintly tuberculose at first; surface colour grey–brown to clay-buff more brownish and rusty or ochraceous stained with age. Lamellae: unequal, crowded, deeply decurrent, forked, not or weakly anastomosing on stipe, pale yellow, red–brown when injured. Stipe: well developed, 4–8 × 0.9–1.8 cm, often curved downwards, almost cylindrical, pale at first staining reddish brown

from the base; rhizomorphs present. Flesh: quickly reddish brown in the stipe, less reactive and slowly saffron yellowish in the cap, smell strong and fruity. Chemical reaction: pileus surface reddish brown with 50 % ammonia solution. Fresh spore deposit: ochraceous only slightly reddish, 5E6–5E7. Spores: $(6.4)6.9\text{--}8.8(9.8) \times (4.6)4.7\text{--}5.4(5.6) \mu\text{m}$ ($n = 34$), elliptical to amygdaliform; apical constriction rare and quite indistinct; Q median 1.52. Hymenial cystidia: relatively small, $50\text{--}73 \times 9\text{--}14 \mu\text{m}$, mostly subcylindrical to fusiform; cheilocystidia frequent; pleurocystidia uncommon. Pileipellis: a cutis to a trichoderm only slightly gelatinized, hyphae $4.8\text{--}7 \mu\text{m}$ wide. Clamp-connections: present throughout.

Ecology and distribution

In groups, always in forest, associated to a wide range of host plant species: *Picea*, *Abies*, *Pinus*, *Betula*, *Fagus*, and *Quercus*. Autumn. Present all over Europe (France, Belgium, Great Britain, The Netherlands, Sweden, Norway, Czech Republic) and in North America (Canada and the USA).

Paxillus cuprinus Jargeat, Gryta, Chaumeton & Vizzini, **sp. nov.**

MycoBank accession number: MB804772.

Paxillus involutus II (Vellinga *et al.* 2012).

P. involutus group IV (Hedh *et al.* 2008).

[Fig 3](#), [Fig 4](#) and [Fig 5](#).

Etymology: from Latin *cuprinus* , derived from  *Κυπρίνος*, of red copper, pileus surface becoming coppery with age.

Holotype: **France**, Haute-Garonne 31, Castanet-Tolosan, alt. approx. 170 m, under *Betula pendula* in city park, leg P. Jargeat, O. Navaud, H. Gryta, 30 November 2010. TL127-0002.

Description

Pileus: (3)5–12(25) cm in diam. At first, subhemispheric, convex to plan-convex then expanding with slightly depressed centre, sometimes more deeply in old specimens, without umbo; surface covered by a faint whitish pruina on the very young specimens, felted and matt when young, slightly sticky in wet weather, glabrous and shiny as leather when dry and with age, frequently with radiating adpressed darker fibrils outwards and, except towards the margin, often areolate or cracked, especially in dry or exposed sites; margin strongly inrolled and felted-tomentose at first, smooth and weakly or not inrolled at maturity, straight or more or less lobate; surface colour grey–brown with an olivaceous shade and some whitish areas, soon becoming ochraceous brown, clay-buff or yellowish olivaceous, finally more uniformly coppery brown or reddish brown, rarely with a vinaceous cast.

Lamellae: unequal, narrow, fairly crowded to crowded (6–13 per cm, two lamellae intermixed by 1–3 lamellulae), decurrent, removable from pileus, often anastomosing and forked towards the stipe; colour at first pale, yellowish white then rusty brown darkening to rusty reddish or reddish coppery with age, staining red–brown when bruised more intensively in young specimens; edges quite obtuse, slightly undulated, paler than faces.

Stipe: $2.7\text{--}5 \times 0.5\text{--}2.5$ cm, rather short in comparison to the pileus diameter, stout but sometimes more slender even very thin, mostly central, cylindric or slightly tapering downwards, dry; whitish background colour, more or less pale pink reddish marbled, often with a distinct light yellow zone at the top, staining reddish brown from the base upward later striated in brown, basal mycelium fairly copious with bundles of pale brown to whitish rhizomorphs mixed with soil.

Flesh: rather thick, firm but soon softened, yellowish in the stipe and in the pileus above the very quickly reddening gills, mainly in young specimens, finally wholly reddish brown to dark-red in just a few hours; smell distinct, fruity; taste mild or slightly bitter.

Chemical reaction: reddish brown to purplish brown with 50 % ammonia solution on pileus surface.

Spore deposit: ochraceous with clearly reddish shade or chocolate brown turning gradually to ochraceous brown more or less olivaceous over time (5E7–6E6–6E7 fading to 5D6).

Spores: holotypus: $(7.0)7.4\text{--}8.8(9.7) \times (4.9)4.9\text{--}5.9(6.1)$ μm ($n = 44$) with median 8.1×5.4 μm ; Q ratio $(1.33)1.39\text{--}1.64(1.66)$ with median 1.50. Based on six collections ($n = 289$): $(7.0)7.2\text{--}9.6(11.5) \times (4.5)4.8\text{--}5.9(6.2)$ μm with median $8.0\text{--}8.6 \times 5.2\text{--}5.5$ μm ; Q ratio $(1.30)1.41\text{--}1.75(2.13)$ with median 1.50–1.63.

Ovoid in frontal view, ovoid to amygdaloid with mostly clearly acute apex in side view and often with a slight depression on the abaxial side towards the top, mostly uniguttulate, yellow–brown, slightly tawny in Melzer's reagent, smooth, apiculus rather short.

Basidia: $(22)27\text{--}46(47) \times 8\text{--}10(11)$ μm ($n = 23$); cylindro-clavate with rather stout sterigmata, four-spored, more rarely two or one-spored.

Hymenial cystidia: $(47)51\text{--}123(137) \times (7)8\text{--}16(19)$ μm , pleuro and cheilocystidia quite similar, mostly clearly distinct from the basidia, of varied shape and size; cylindrical-tapered, ventricose with a long neck, irregularly flexuose to lageniform with acute to subcapitate apex, at times also broadly fusiform to saccate but more often relatively narrow, abruptly constricted to the connection with basal hyphae, hyaline or with a brown content sometimes only on the upper part, with no incrustations in 5 % KOH. Hyaline or brownish cystidioid elements, clearly distinct from the basidioles, versiform, similar to caulocystidia, but smaller ($29\text{--}49 \times 4\text{--}8$ μm), are sometimes found on the edge of lamellae.

Hymenophoral trama: subregular, slightly divergent, mediostratum with hyaline to brown pigmented hyphae, $5.5\text{--}17.5$ μm wide.

Pileipellis: a cutis to an intricate trichoderm not distinctly or poorly gelatinized with septate, sometimes fasciculate hyphae, $(4)4.6\text{--}7.7(8.1)$ μm wide, with brown intracellular pigment and free terminal elements poorly differentiated.

Stipitipellis: superficial hyphae brown yellowish, $3.9\text{--}9.1$ μm wide, caulocystidia smaller than hymenial cystidia, $(44\text{--}72 \times 8\text{--}9)$ μm , versiform, cylindrical-tapered, cylindrical-flexuose, capitate, sometimes in part thick-walled, hyaline to brownish. Fertile caulohymenium is present on the upper part of stipe.

Stipititrampa: made up of $10\text{--}32$ μm hyphae wide.

Rhizomorphs: superficial layer with narrow hyphae 2.8–5.2 µm wide, dotted with microcrystals, central part with larger 8.8–17.5 µm thick-walled hyphae wide.

Clamp-connections: present at all septa.

Ecology and distribution

In groups or sometimes by two or three connate basidiomes, always in bright places: lawns, parks, river banks, forest edges, and along paths. Growing in association with *Betulaceae* (*Betula*, *Alnus*, *Corylus*, *Ostrya*) both in urban areas and in more natural sites. Late summer and autumn.

Widespread and probably fairly common in Europe (France, Germany, Italy, Sweden), also present in Asia (Iran) and North America (introduced in Northwest USA, California, Washington).

Additional specimens examined: **France:** Haute-Garonne 31, Castanet-Tolosan, alt. approx. 170 m, under *Betula pendula* in city park, leg O. Navaud, 15 October 2013, Cas12.5. Saône-et-Loire 71, Saint-Vincent-en-Bresse, alt. approx. 210 m, in a public lawn under *B. pendula*, leg S. Pitois, 26 November 2008, SVB08.1; Pyrénées-Orientales 66, Prats-de-Mollo, alt. approx. 925 m, roadside in forest under *B. pendula*, leg P. Jargeat and H. Gryta, 03 October 2010, CA10.A1; Gers 32, Eauze, alt. approx. 145 m, river bank under *Alnus glutinosa*, leg J.P. Chaumeton, 09 November 2011, B111101; Haute-Garonne 31, Toulouse, alt. approx. 150 m, in a public lawn under *Cedrus* sp., not far from *B. pendula*, leg J.P. Chaumeton, 11 November 2011, B111114; Tarn 81, Arfons, alt. approx. 750 m, mixed forest with *B. pendula* and *Fagus sylvatica*, leg P. Jargeat and H. Gryta, 12 October 2012, MN12.08; Haute-Garonne 31, Castanet-Tolosan, alt. approx. 150 m, in a public lawn under *B. pendula*, leg P. Jargeat, H. Gryta and J.P. Chaumeton, 15 October 2012, B121005; Hautes-Pyrénées 65, Cantaous, alt. approx. 590 m, in a forest path under *Corylus avellana*, leg J.P. Chaumeton, 16 October 2012, B121007.

Italy: Vezzano S.C., Reggio nell' Emilia (Emilia Romagna), Case di Casino, Monte Duro, alt. approx. 500 m, in a mixed forest of deciduous woods with *Quercus pubescens*, *Fraxinus ornus*, *Ostrya carpinifolia*, *Castanea sativa*, on a small slope at the side of a wide path, leg. G. Simonini, 21 October 2012, TO AVP164 (GS10076).

Selected icons: [Ludwig \(2000\)](#) pl. 61.2B. The shape of basidiomes, the olivaceous colour and the whitish discolouration of the young specimen are in accordance with *Paxillus cuprinus*. The description by the same author (s.n. *Paxillus involutus*) is more collective. [Sowerby's \(1797\)](#), pl. 56) colour plate of '*Agaricus contiguus*' also gives a good representation of *P. cuprinus*.

Key to species of the *Paxillus involutus* complex in Europe

1. Green reaction on the pileus with concentrated ammonia (more obvious on the slimy part). Pileus surface often viscid to glutinous and usually brightly coloured with yellow, orange or fulvous shade. Spore print in most cases greenish tinged *Paxillus ammoniavirescens*(=*Paxillus validus*).

1*. No green reaction on the pileus with concentrated ammonia. Pileus surface slightly slimy only in wet weather and with duller colours. Fresh spore print reddish without a greenish shade 2.

2. Basidiome fairly large, with pileus up to 30 cm diameter, often growing in clusters. Fresh spore print deep reddish brown to vinaceous brown. Spores broadly ellipsoid (*Q* value 1.38–1.54), only the largest nearly subamygdaliform *Paxillus obscurisporus*.

2*. Basidiome smaller and slender, with pileus up to 12(20) cm, scattered or connate by 2 or 3. Fresh spore print lighter, ochraceous with reddish shade. Spores ovoid or ellipsoid or elliptic-amygdaliform **3**.

3. In forests, typically in shaded places. Associated with a large range of trees, conifers or hardwoods. Pileus often umbonate with variable colours: grey–brown, rusty brown, ochraceous brown, olivaceous brown, sometimes whitish. Spores ellipsoid, ovoid or ellipsoid–amygdaliform, with apical constriction inconstant to rare *P. involutus*.

3*. In bright places, in urban as well as natural areas. Associated with *Betulaceae* (*Betula*, *Alnus*, *Corylus*, *Ostrya*). Pileus with olivaceous shade when young and coppery brown or reddish brown in age. Spores ellipsoid–ovoid to amygdaliform with a constant to frequent apical constriction *Paxillus cuprinus*.

Discussion

In this study, we combined molecular and taxonomic approaches and we included available data from the literature and from sequence databases as much as possible to clearly characterize species belonging to the *Paxillus involutus* complex.

The phylogenetic analysis conducted with three genes (ITS, *tef1*- α , and *gpd*) and with the concatenated data set revealed four lineages, in accordance with the results obtained by [Hedh et al. \(2008\)](#) with a set of five different genes (*hydA*, *actA*, *rabA*, *gpiA*, and β -*tubA*) and [Vellinga et al. \(2012\)](#). As intralineage genetic diversity was always lower than interlineage genetic diversity, the delimitation of the four species is very well supported. The *tef* and *gpd* trees do not recover the same topological relations as the ITS or concatenated trees but a similar result was also obtained with the gene *hydA* ([Hedh et al. 2008](#)), that clusters *Paxillus cuprinus*, *Paxillus obscurisporus*, and *Paxillus ammoniavirescens* while *P. involutus* is more distant. In fact, these genes encode for proteins and most of the variability is located in their introns. Introns accumulate mutations and have an accelerated, independent evolution rate. Furthermore, proteins may have diverged at different rates, possibly due to changes in selection pressure.

Two of the phylogenetic species are identified according to *P. obscurisporus* and *P. ammoniavirescens* type specimens sequences.

We only studied two *P. obscurisporus* collections, so it was not possible to go further than [Hahn & Agerer](#) into the description of that species (1999). Like these authors, we observed lead-grey to ochre–grey, large and massive basidiomes, growing in clusters. The spore deposit was dark, the spores were broadly ellipsoid and only the largest ones had a subamygdaloid shape. The results of our *P. obscurisporus* spore and cystidia measurements are totally in accordance with those of [Hahn & Agerer \(1999\)](#). This species seems to be preferentially associated to deciduous trees but [Hahn & Agerer \(1999\)](#) indicate it under *Abies procera*, suggesting a broader host range. It seems to be heliophilous since it grows in city parks and public lawns, and it was found in forest edges in natural conditions. It is present in northern France ([Moreau et al. 2013](#)), northern Europe ([Hedh et al. 2008](#)), and in Chinese mountains ([Binder & Hibbett 2006](#)). In the southwest of France, it was found in relatively cool submountainous areas with significant rainfall. Thus, a mesophilic or psychrophilic trend is supposed for this species.

According to [Dessi & Contu \(1999\)](#), *P. ammoniavirescens* is easily identified from the green reaction of its pileus surface with ammonia and is characterized by relatively small basidiomes (5–13 cm), yellow–green tinges, light-brown spore prints, chrome-yellow flesh and growth in clusters.

It was collected in Mediterranean maquis and forests. However, *Paxillus validus*, later shown to be *P. ammoniavirescens* ([Vellinga et al. 2012](#)), is larger (7–20 cm), more robust, more reddish brown and was collected in city parks in Germany ([Hahn & Agerer 1999](#)). Our specimens belong to either morphological type, with either large basidiomes from urban sites or slender basidiomes from river banks or forest edges. Spore shape is variable and statistical analyses emphasize a high diversity of spore sizes. In fact, some of our collections have the smallest spores of all samples, while a collection from Morocco ([Vizzini 2009](#)) has large spores ($7.3\text{--}9.7 \times 5.2\text{--}6.0$) and our Cas12.1 collection has the largest spores of all our sampling: $(7.5)7.7\text{--}10.6(11.6) \times (4.8)4.9\text{--}6(6.3) \mu\text{m}$ (mean of $9 \times 5.3 \mu\text{m}$).

Morphological variability within this species has led to some misinterpretations. *Paxillus vinosofractus* nom. prov. ([Dessì & Contu 1999](#)) looks very much like *P. ammoniavirescens* on a morphological basis ([Vizzini 2009](#)) and *P. involutus* f. *subrubicundulus* Bon ([Bon & Van Haluwyn 1981](#)) appears to be the same as *P. ammoniavirescens* since it has vivid colours, a bright-coloured pileus, yellow gills, a greenish spore print, and ellipsoid spores. Unfortunately, we failed to amplify sequences from the holotype material of this taxon.

Pure *P. ammoniavirescens* cultures were obtained from large basidiomes and slender basidiomes. In all cases, mycelia grow fast and produce brown droplets. In contrast, [Hahn & Agerer \(1999\)](#) observed a slow growth (1 cm per month) when they grew this species. To explain this discordance, we can hypothesize that they did not observe enough cultures to access the whole range of variability among *P. ammoniavirescens* cultures.

Paxillus ammoniavirescens is associated with a very large range of deciduous trees belonging to *Fagaceae*, *Salicaceae*, *Betulaceae* (this study), and even *Eucalyptus* and *Cistus* ([Dessì and Contu, 1999](#) and [Vizzini, 2009](#)) as well as coniferous trees such as *Pinus*, *Cedrus* (this study), and *Abies* ([Dessì & Contu 1999](#)). *Paxillus ammoniavirescens* may be heliophilous since it is always found in open areas like city parks and gardens (*P. validus* form) and in natural conditions, it grows in bright places such as maquis ([Dessì & Contu 1999](#)), forest edges, river banks or meadows (*P. ammoniavirescens* form). It has the largest ecological range, from cold and wet climate in Sweden ([Hedh et al. 2008](#)) to Mediterranean hot and dry conditions in Italy (reported from Sardinia by [Dessì & Contu \(1999\)](#)), Morocco ([Vizzini 2009](#)), and the South East of France ([Vellinga et al., 2012](#) and [Moreau et al., 2013](#)).

A third lineage including the ATCC 200175 strain, whose complete genome is now sequenced (<http://genome.jgi-psf.org/Paxin1/Paxin1.home.html>), was previously assigned to *P. involutus* s. st. ([Hedh et al., 2008](#) and [Vellinga et al., 2012](#)). Pale variants (*P. involutus* f. *eburneus* and a whitish collection from the UK) belong to that lineage ([Gelardi et al., 2011](#) and [Vellinga et al., 2012](#)) and *Paxillus albidulus* ([Sütara 1992](#)), described as a small-sized species occurring in coniferous forests but not yet sequenced, is also most probably a white form of *P. involutus*. This species is characterized by its umbonate pileus. Spores are ellipsoid with sometimes a slight depression on the dorsal side near the apex. No brown droplets are observed in *P. involutus* cultures, as mentioned by [Hahn & Agerer \(1999\)](#). It occurs under hardwoods and coniferous trees but only in closed areas such as forests. It may be a sciaphilous species and this could partially explain its absence in urban areas where large wooded parts are scarce.

The *P. involutus* concept is based on [Batsch's icône \(1786\)](#). We consequently chose our Can12.1 collection, which belongs to that lineage and matches [Hahn & Agerer's](#) description of *P. involutus* (1999), as the epitype for this species.

The fourth lineage corresponds to phylogenetic species IV in [Hedh et al. \(2008\)](#) and *P. involutus* II in [Vellinga et al. \(2012\)](#) and had never been described before. Based on the morphological description of our 17 collections, this species was named *P. cuprinus* since the colour change from grey–brown–olivaceous to coppery with age is an important feature to distinguish it. Moreover, it is characterized by spores with a slight depression on their dorsal side near the apex. On average, it has larger spores than the other three species, with a low variability confirmed by statistical analysis. In pure cultures, *P. cuprinus* mycelia grow slowly and produce brown droplets. All our *P. cuprinus* collections (from 11 different sites) were found near *Betulaceae*, including *Alnus*. All the literature data also mention it in the vicinity of *Betulaceae* ([Hedh et al., 2008](#), [Bahram et al., 2012](#) and [Vellinga et al., 2012](#)). This suggests a preference for this plant family. When associated with *Alnus*, *P. cuprinus* could be mistaken for *Paxillus rubicundulus* but *P. rubicundulus* has smaller spores ([Orton 1969](#)). *Paxillus cuprinus* seems to be heliophilous since it was always found in bright places, frequently in urban areas.

Despite a consistent collecting effort, in very different environments, and numerous sequences from other studies, all specimens and sequences analysed in this study are only assigned to one of the four species, reinforcing the hypothesis that there are only four species in the *P. involutus* complex in Europe. All the groups or species described so far should belong to one of those four species. For example, the three intersterility groups described by [Fries \(1985\)](#) as a ‘forest type’ mainly associated to coniferous trees and two ‘park types’ growing in tight groups in anthropic environments, with connate stipes, appear to correspond to woodland *P. involutus* and *P. obscurisporus* and *P. ammoniavirescens*, respectively. The four *Paxillus* species are common in Europe but they are also reported in North America, as native species such as *P. involutus* or as introduced species such as *P. cuprinus* ([Vellinga et al. 2012](#)), in Asia (*P. cuprinus* and *P. obscurisporus*, [Bahram et al., 2012](#) and [Binder and Hibbett, 2006](#)) and according to [Vizzini \(2009\)](#), *P. ammoniavirescens* was found in Africa (Morocco). The presence of the North American *Paxillus vernalis* ([Watling 1969](#)) in Europe was reported ([Jarosch and Bresinsky, 1999](#), [Bresinsky, 2006](#) and [Kibby, 2008](#)) but identification was based on an erroneous concept of this species, established by [Jarosch & Bresinsky \(1999\)](#). In fact, sequences from one of those collections (Pi1 [AF167690](#), Pi12 [AF167691](#), PiM1 [AF167692](#), PiM4 [AF167693](#), PiM2 [AF167694](#), [Jarosch & Bresinsky 1999](#)) belong to the *P. obscurisporus* phylospecies (this study) and the morphological descriptions suggest that the two other collections could belong to *P. ammoniavirescens*, *P. obscurisporus* or *P. cuprinus*.

When comparing the genetic diversity of *P. cuprinus* and *P. involutus* on the one hand and *P. ammoniavirescens* and *P. obscurisporus* on the other hand, the ITS genetic divergence (*K*) is below 3 %, the admitted threshold to identify fungal species by barcoding ([Peay et al., 2008](#) and [Begerow et al., 2010](#)). Genetic divergence was also below 3 % when comparing *Tricholoma scalpturatum* and *Tricholoma inocybeoides* (*K* = 1.98 %) or *Tricholoma argyraceum* and *Tricholoma cingulatum* (*K* = 1.78 %) ([Jargeat et al. 2010](#)). Thus, in the two very distant genera *Tricholoma* and *Paxillus* but also in genera such as *Cortinarius* ([Frøslev et al. 2007](#)) or *Alnicola* ([Rochet et al. 2011](#)), the interspecific level is reached for genetic divergence values below 3 %. As mentioned by [Osmundson et al. \(2013\)](#) or [Hibbett et al. \(2011\)](#), a unique sequence similarity cut-off for species delimitation, as is often used in molecular ecology studies, may introduce significant errors in species diversity estimates. A 1–2 % divergence threshold appears as a good compromise.

All our observations and results indicate a strong stability in morphological features and host association for *P. cuprinus* while *P. ammoniavirescens* is highly variable, associated to a very large host range and is present through very different environmental conditions all over Europe. These observations correlate with the genetic results that highlight a low intraspecific genetic diversity in *P. cuprinus*, contrasting with the high genetic variability of *P. ammoniavirescens*. These differences

are supported by intraspecific genetic variations (π) calculated on a concatenated data set (1893 bp) generated from five other genes ([Hedh et al. 2008](#)). Actually, our π values are in accordance with those of 0.0009 and 0.01 calculated for group IV (*P. cuprinus*) and for group III (*P. ammoniavirescens*), respectively ([Hedh et al. 2008](#)). We can hypothesize that these two species have different dispersal and colonisation patterns in connection with adaptive ability. *Paxillus ammoniavirescens* could have features of pioneer and/or ruderal species that invest in sexual reproduction and spore production to colonise open areas, leading to a great genetic diversity. In contrast, *P. cuprinus*, like host-specific species such as *Tricholoma populinum* ([Gryta et al., 2006](#) and [Grubisha et al., 2012](#)) or *Suillus bovinus* and *Suillus pictus* ([Dahlberg and Stenlid, 1990](#) and [Hirose et al., 2004](#)) may have a more competitive strategy, which promotes the local persistence of mycelium attached to a specific host.

Conclusion

This study enabled us to build a clear framework for the *Paxillus involutus* complex and to reveal a few ecological characteristics: *Paxillus cuprinus* seems to have a preference for *Betulaceae*, *P. involutus* appears to be restricted to forests, under a dense crown cover, *Paxillus obscurisporus* can be related to cool and wet climates and *Paxillus ammoniavirescens* is likely to have a wide range of hosts and a worldwide distribution. For this species, the diversity at the genetic level is supported by morphological and ecological variabilities, which are often a major obstacle when telling different species apart. The genus *Paxillus* deserves further investigations in order to characterize and describe other species (*Paxillus rubicundulus*, *Paxillus vernalis* in North America) and population studies would help to better understand the biology and dispersal strategies of the different species.

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